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Dreier, B ; Plückthun, A

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Chapter 21

Ribosome Display: A Technology for Selecting and Evolving Proteins from Large Libraries

Birgit Dreier and Andreas Plückthun

Abstract

The selection and concomitant affinity maturation of proteins to bind to user-defined target molecules have become a key technology in biochemical research, diagnostics, and therapy. One of the most potent selection technologies for such applications is ribosome display. It works entirely *in vitro*, and this has two important consequences. First, since no transformation of any cells is required, libraries with much greater diversity can be handled than with most other techniques. Second, since a library does not have to be cloned and transformed, it is very convenient to introduce random errors in the library by PCR-based methods and select improved binders. Thus, a true directed evolution, an iteration between randomization and selection over several generations, can be conveniently carried out, e.g., for affinity maturation. Ribosome display has been used successfully for the selection of antibody fragments and other binding proteins, such as *D*esigned *A*nkyrin *R*epeat *P*roteins (DARPins).

Key words: Ribosome display, *In vitro* selection, *In vitro* translation, Designed ankyrin repeat proteins, Affinity maturation

1. Introduction

In order to select and evolve proteins or peptides from a library to bind to any chosen target of interest, different selection strategies can be applied. All technologies have in common that the phenotype (peptide or protein scaffold) is physically linked to the genetic information (DNA or mRNA). We term "*in vivo*" those technologies that require transformation of cells with a library, as needed, for example, in phage display (1). In contrast, "*in vitro*" technologies do not require any transformation of cells with the library. Examples are ribosome display (2, 3) or mRNA display (4, 5). In each case, selection of highly specific binders is performed over multiple rounds of selection, starting from a library of peptides or proteins with a natural or designed scaffold (Fig. 1).

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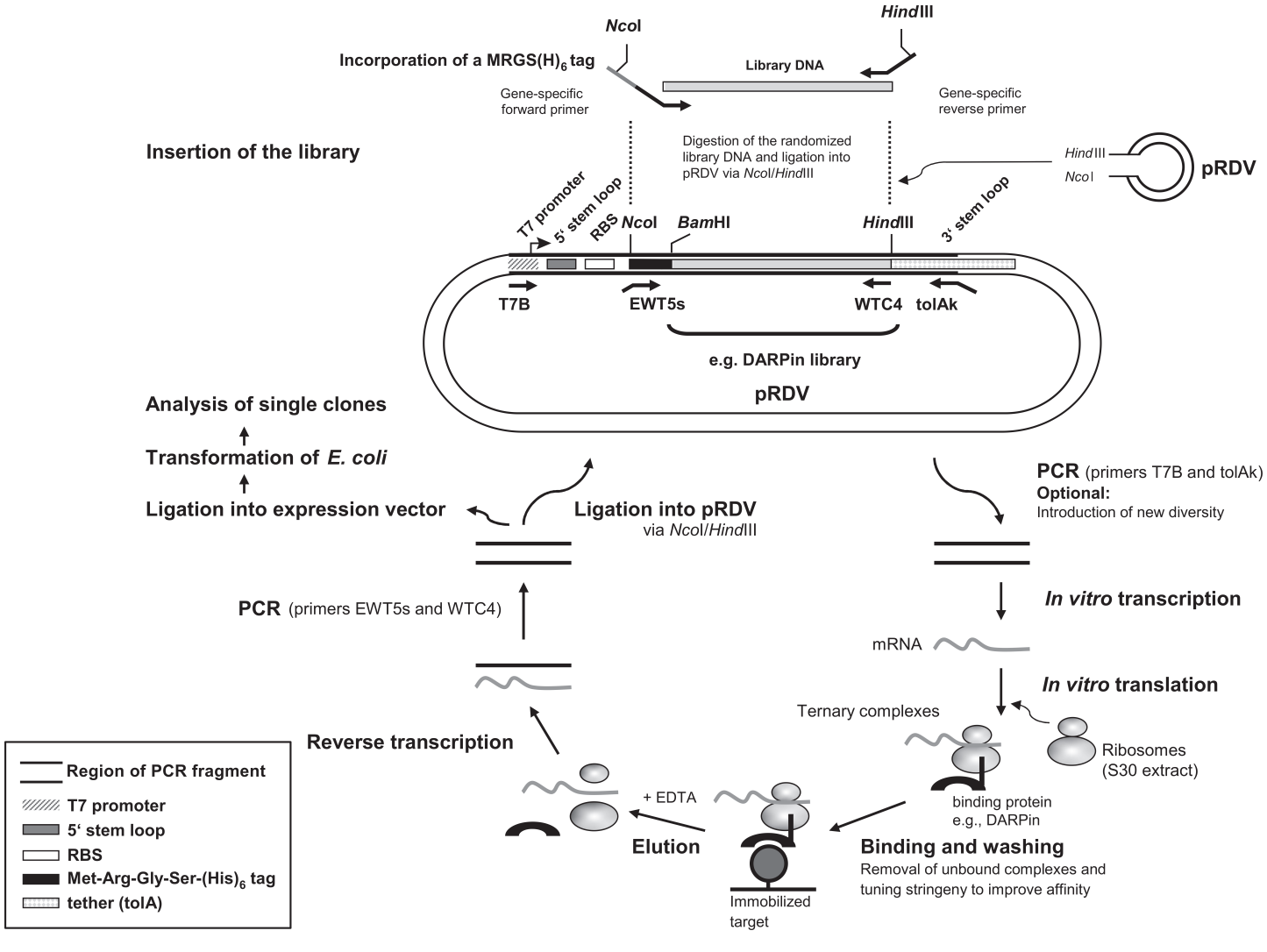
Incorporation of a MRGS(H)6 tag
NcoI
HindIII
Library DNA
Gene-specific forward primer
Gene-specific reverse primer
Insertion of the library
Digestion of the randomized library DNA and ligation into pRDV via NcoI/HindIII
HindIII
NcoI
pRDV
T7 promoter
5' stem loop
RBS
NcoI
BamHI
HindIII
3' stem loop
T7B
EWT5s
WTC4
tolAk
e.g. DARPin library
pRDV
Analysis of single clones
Transformation of E. coli
Ligation into expression vector
Ligation into pRDV
via NcoI/HindIII
PCR (primers T7B and tolAk)
Optional:
Introduction of new diversity
PCR (primers EWT5s and WTC4)
In vitro transcription
mRNA
In vitro translation
Ternary complexes
Ribosomes
(S30 extract)
Reverse transcription
+ EDTA
Elution
binding protein
e.g., DARPin
Binding and washing
Removal of unbound complexes and tuning stringeny to improve affinity
Immobilized target
Region of PCR fragment
T7 promoter
5' stem loop
RBS
Met-Arg-Gly-Ser-(His)6 tag
tether (tolA)

Fig. 1. Scheme of the ribosome display cycle, illustrated for selection of high-affinity DARPins. In ribosome display all steps of the selection are performed *in vitro*. The cycle begins with a DNA library (*top*) in the form of a PCR fragment encoding a library of the protein of interest. This cassette is ligated into a vector *in vitro*, which provides a promoter and ribosome binding site. The ORF of interest (*light gray*) is fused to an additional protein region (the "spacer" or "tether," *checkered white*). This tether or spacer, here used as an unstructured region from the *E. coli* TolA protein, has the sole function of allowing the protein domain of interest to emerge from the ribosomal tunnel. A PCR is then carried out from the promoter to the middle of the tether. Importantly, the PCR fragment does not encode a stop codon at the end. Each member of the library pool is then transcribed from double-stranded DNA into mRNA and is subsequently translated by the ribosomes present in the S30 extract, leading to ternary complexes consisting of ribosomes, mRNA, and the DARPin encoded by that particular mRNA. Since there is no stop codon on the mRNA, the protein is not released from the ribosome. It is believed to be still covalently attached to the tRNA within the ribosome, with the tether in the tunnel, and the domain of interest outside and already folded. Selection can be achieved by binding the protein–ribosome–mRNA complexes to the desired immobilized target, followed by removal of unbound or nonspecifically bound protein by stringent washing. Affinity can be increased by addition of an excess of nonlabeled target (off-rate selection) (see Subheading 3.5.4). Particular selectivity in binding can be achieved by adding an unwanted target as a competitor. Selection for other properties, such as stability, requires other selection pressures at this step (see Note 25). Binders can be easily recovered by destruction of the protein–ribosome–mRNA complex using EDTA and recovery of the genetic information of the binders by RT-PCR using the inner primers WTC4 (annealing to the sequence encoding the C-terminus of the DARPin sequence which can be replaced by a primer specific for other library folds) and EWT5s [pRDV-specific primer overlapping with the ribosome binding site (RBS) and beginning of the Met-Arg-Gly-Ser-$(His)_6$ tag]. The inner primer set is used to amplify the selected clones, which often is not possible with the outer primer set due to incomplete synthesis or degradation of the mRNA. For further selection rounds, the PCR product pool is subcloned into pRDV via the restriction endonucleases *Nco*I and *Hin*dIII, followed by a second PCR with the outer primers T7B and tolAk. T7B introduces the T7 promoter sequence and part of the stabilizing 5′ stem loop, sequences that are part of the pRDV vector. The tolAk primer binds in the sequence of the tolA spacer region and introduces a stabilizing 3' stem loop. If further diversity is required an error-prone PCR can be included at this step. The amplified PCR product then serves as template for *in vitro* transcription, initiating the next round of selection. At the end of the selection rounds (typically, 2–5), the resulting PCR product pool can be directly subcloned via the restriction endonucleases *Bam*HI and *Hin*dIII into an expression vector in order to screen for binders.

Ribosome display has some major advantages compared to *in vivo* selection strategies. First, the library size is not restricted to limitations in transformation efficiency, which for *Escherichia coli* usually is 10^9–10^{10} per microgram of DNA and significantly lower when a ligation mixture is used. In ribosome display, library size is only limited by the number of ribosomes present in the *in vitro* translation and can be as high as 10^{12}–10^{14}. Second, while in the living cell protein biosynthesis and folding occur in a given environment, the environment using an *in vitro* selection system can be manipulated and optimized for expression, folding, and stability of the library members. This can be exploited for stability selection (6, 7). Third, the diversity of the library members can be easily manipulated at any selection step by introduction of additional mutations using DNA shuffling (8) and/or error-prone PCR (9). This is perhaps the factor of greatest practical utility. In other technologies, after each randomization step, a new library needs to be ligated and transformed. In ribosome display, an additional randomization step merely alters the method of library amplification. Therefore, ribosome display is particularly suited for directed evolution projects over many generations.

Ribosome display selections have been first exploited for peptides (10), but the true advantage of directed evolution by using error-prone PCR methods was only borne out with proteins, and antibody scFv fragments (3, 7, 11, 12) were the first protein used. By using very stringent selections for affinity, antibody scFv fragments have been evolved to affinities as high as 5 pM or even 1 pM (12, 13). By choosing an appropriate selection pressure, properties other than affinity could be optimized, such as, for example, stability (7).

Over the last years also new protein scaffolds, with more desirable biophysical properties than antibody fragments, have been investigated. Among the most promising scaffolds are the *D*esigned *A*nkyrin *R*epeat *P*roteins (DARPins), which are devoid of disulfide bonds, highly soluble, and highly stable, and therefore achieve high expression levels in *E. coli* (14). They also fold well in the *in vitro* translation inherent in ribosome display. Using ribosome display DARPins have been evolved to bind various targets with affinities all the way down to the picomolar range (15–20). In general, probably because of their robust *in vitro* folding, DARPins are enriched over fewer rounds than antibody fragments.

Taken together, ribosome display is an ideal tool to select and evolve proteins with predefined binding properties from large libraries.

2. Materials

2.1. General

1. 96-well Maxisorp plates (Nunc, No. 442404) or strips.
2. Adhesive plate sealers (Thermo Scientific, No. AB-0580).

3. Sterile, RNase-free ART filter tips (Molecular Bio Products).
4. Sterile, RNase-free HydroLogix 1.5 and 2.0-ml tubes (Molecular Bio Products, No. 3448 or No. 3434).
5. Roche high pure RNA isolation kit (Roche, No. 11828665001).
6. illustra MicroSpin™ G-50 Columns (GE Healthcare, No. 27-5330-01).
7. NucleoSpin® Extract II DNA purification kit (Macherey-Nagel, No. 740609.50).

2.2. Reagents for Selection (See Note 1)

1. Tris-buffered saline (TBS): 50 mM Tris, 150 mM NaCl; adjust pH to 7.4 with HCl at 4°C; filter through 0.22 μm.
2. TBST: TBS containing 0.05% Tween-20.
3. Stock solutions for wash buffer (WB) and elution buffer (EB): 2 M Tris-acetate; adjust pH to 7.5 at 4°C with acetic acid, 5 M NaCl, 2 M magnesium acetate, 250 mM EDTA; adjust pH to 8.0 by NaOH addition. Sterile filter all solutions. For alternative buffer composition see Note 2.
4. WB/Tween-20 (WBT): 50 mM Tris-acetate pH 7.5, 150 mM NaCl, 50 mM magnesium acetate, 0.05% Tween-20; adjust pH to 7.5 with acetic acid at 4°C; filter through 0.22 μm.
5. EB: 50 mM Tris-acetate pH 7.5, 150 mM NaCl, 25 mM EDTA; adjust pH to 7.5 with HCl at 4°C; filter through 0.22 μm.
6. *Saccharomyces cerevisiae* RNA (BioChemica, No. 83847): dissolve to 25 μg/μl in H_2O, aliquot and store at −20°C.
7. 10% BSA in H_2O: filter through 0.22 μm and store at −20°C.
8. Neutravidin and/or streptavidin (Pierce, No. 31000 or No. 21125): 1.2 mg/ml (20 μM) in TBS and store at −20°C.
9. Streptavidin-coated magnetic beads (MyOne T1; Invitrogen, No. 65602).
10. Reagents for biotinylation of the target: either for chemical biotinylation a NHS-biotin reagent [e.g., from Pierce EZ-link™ SulfoNHS-LC-biotin (No. 21335)] or for enzymatic biotinylation of an AviTag using the *E. coli* biotinylation enzyme BirA (21) (reagents from Avidity).

2.3. Reagents for mRNA Cleanup After In Vitro Transcription

1. 6 M LiCl; filter through 0.22 μm pores.
2. 3 M sodium acetate; filter through 0.22 μm pores.
3. 70% EtOH diluted with H_2O and 100% EtOH; filter through 0.22 μm pores.
4. illustra MicroSpin™ G-50 Columns (GE Healthcare, No. 27-5330-01).
5. DNAseI (10 U/μl; Roche, No. 04716728001).

2.4. Reagents for Reverse Transcription, PCR and Cloning

1. Primer dissolved to 100 µM in H_2O; aliquot and store at –20°C.
   EWT5s: 5′-TTCCTCCATGGGTATGAGAGGATCG-3′
   WTC4: 5′-TTTGGGAAGCTTTTGCAGGATTTCAGC-3′
   T7B: 5′-ATACGAAATTAATACGACTCACTATAGGGAGA CCACAACGG-3′
   tolAk: 5′-CCGCACACCAGTAAGGTGTGCGGTTTCAG-TTGCCGCTTTCTTTCT-3′
2. AffinityScript™ Multiple Temperature Reverse Transcriptase (50 U/µl; Stratagene, No. 600107) and 10× buffer; see Note 3.
3. 100 mM DTT in H_2O; aliquot and store at –20°C.
4. RNasin® Ribonuclease Inhibitor (20–40 U/µl; Promega, No. N2115).
5. $Vent_R$® DNA Polymerase (2 U/µl; New England Biolabs, No. M0254S) and 10× Thermopol buffer; see Note 4.
6. Platinum® Taq DNA Polymerase (5 U/µl; Invitrogen, No. 10966083) and 10× polymerase buffer.
7. dNTPs: 5 mM each (Eurogentec, No. NU-0010-10); aliquot and store at –20°C.
8. Nucleotide analogs dPTP and 8-oxo-dGTP (Jena Biosciences) at a concentration of 100 µm.
9. Dimethyl sulfoxide (DMSO; Fluka, 41640).
10. Restriction endonucleases: *Bam*HI (20 U/µl; No. R0136L), *Hin*dIII (20 U/µl; No. R0104L), *Nco*I (10 U/µl; No. R0193L), and 10× buffer all from New England Biolabs.
11. T4 DNA ligase (5 U/µl; Fermentas, No. EL0014) and 10× ligase buffer.
12. Ribosome display vector pRDV (GenBank accession code AY327136; please note the revised sequence) (16).

2.5. Reagents for In Vitro Transcription

1. T7 RNA polymerase (20 U/µl; Fermentas, No. EP0111); see Note 5.
2. RNasin® Ribonuclease Inhibitor (20–40 U/µl; Promega, No. N2115).
3. 100 mM DTT in H_2O; aliquot and store at –20°C.
4. T7 RNA polymerase buffer (5×): 1 M HEPES, 150 mM magnesium acetate, 10 mM spermidine, 200 mM DTT; adjust pH to 7.6 with KOH; aliquot and store at –20°C.
5. 50 mM NTP mix: 50 mM adenosine 5′-triphosphate (ATP; Sigma-Aldrich, No. A2383), 50 mM uridine 5′-triphosphate (UTP; Sigma-Aldrich, No. U6625), 50 mM guanosine 5′-triphosphate (GTP; Sigma-Aldrich, No. G8877), 50 mM cytidine 5′-triphosphate (CTP; Sigma-Aldrich, No. C1506) in H_2O; aliquot and store at –20°C.

2.6. Reagents for In Vitro Translation

1. Protein disulfide isomerase (PDI; Sigma-Aldrich, No. P3818): 22 µM in H_2O; aliquot and store at −80°C.
2. Heparin (Sigma-Aldrich, No. H4784) stock solution: 200 mg/ml heparin in H_2O (do not filter); aliquot and store at −20°C.
3. Methionine (Sigma-Aldrich, No. M9625): 200 mM l-methionine in H_2O (do not filter); aliquot and store at −20°C.
4. STOP mix: 1 ml WBT buffer/0.5% BSA plus 12.5 µl heparin stock solution (see above).

2.6.1. S30 Extract

1. *E. coli* strain MRE600 (ATCC 29417) (22) lacking ribonuclease I activity.
2. Incomplete rich medium: 5.6 g KH_2PO_4, 28.9 g K_2HPO_4, 10 g yeast extract, 15 mg thiamine for 1 l medium. Autoclave and add 50 ml 40 % glucose (w/v) and 10 ml 0.1 M magnesium acetate, both sterile filtered.
3. S30 buffer: 10 mM Tris-acetate (pH 7.5 at 4°C), 14 mM magnesium acetate, 60 mM potassium acetate. Chill to 4°C before use.
4. Preincubation mix (must be prepared directly before use): 3.75 ml 2 mM Tris-acetate (pH 7.5 at 4°C), 71 µl 3 M magnesium acetate, 75 µl amino acid mix (10 mM of each of the 20 amino acids; Fluka, No. LAA21), 300 µl 0.2 M ATP, 50 U pyruvate kinase (Fluka, No. 83328), 0.2 g phosphoenolpyruvate trisodium salt (Fluka, No. 79435); add to 10 ml H_2O.

2.6.2. PremixZ

1. Set up premixA (the final concentration will be fivefold lower in the final volume of the *in vitro* translation reaction; see Subheading 3.4): 250 mM Tris-acetate (from a 2 M stock solution, pH 7.5 at 4°C), 18 µM anti-ssrA oligonucleotide (5′-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA-3′) from a 200 µM stock solution, 1.75 mM of each amino acid except for methionine, 10 mM ATP from an 1 M stock solution, 2.5 mM GTP from a 0.2 M stock solution, 5 mM cAMP (Sigma-Aldrich, No. A6885) from 0.4 M stock solution, 150 mM acetyl phosphate (Sigma-Aldrich, No. A0262) from 2 M stock solution, 2.5 mg/ml *E. coli* tRNA from strain MRE600 (Roche, No. 10109541001) from a 25 mg/ml stock solution, 0.1 mg/ml folinic acid (Sigma-Aldrich, No. 47612) from 10 mg/ml stock solution.
2. Set up an *in vitro* translation reaction (see Subheading 3.4), and use the above premixA but titrate the optimal concentration of the following components for the final premixZ composition to achieve optimal performance of each newly generated S30 extract. Optimize the final concentrations in the order shown:

Magnesium acetate (MgAc) usually in the range of 7–15 mM from a 0.2 M stock solution, potassium glutamate (KGlu) usually in the range of 180–220 mM from a 2 M stock solution, and PEG-8000 usually in the range of 5–15% (w/v) from a 40% stock solution. Adjust the premixA with the optimal composition of MgAc, KGlu, and PEG to obtain the premixZ. Aliquot the premixZ and flash-freeze in liquid nitrogen. Long-time storage should be at –80°C, but the premixZ is stable for several months at –20°C and can be frozen several times. If not noted otherwise, reagents were purchased from Sigma-Aldrich.

2.6.3. β-Lactamase Assay

Used to test the activity of the S30 extract and optimization of the premixZ.

1. Prepare β-lactamase mRNA from the pRDV template DNA encoding the double Cys → Ala mutant of β-lactamase (23) using PCR with the T7B and tolAk primers (Fig. 1) (see Subheading 3.2), followed by *in vitro* transcription and purification of mRNA (Protocol 3.2 and 3.3).
2. Set up *in vitro* translation reactions containing 2 µg RNA, 0.5 µl 200 mM methionine, 10 µl S30 extract, 8.2 µl premixZ and add to 22 µl H_2O. For optimization of the activity of the S30 extract use premixA and adjust the concentration of magnesium acetate, potassium acetate, and PEG-8000.
3. Incubate at 37°C for 10 min.
4. Add 88 µl STOP mix
5. Use 5 µl of stopped *in vitro* translation for the activity assay with the chromogenic substrate nitrocefin (Glaxo Research No. 87/312, obtained from Oxoid No. SR0112) (24).
6. Dilute nitrocefin 1:20 in β-lactamase buffer (100 mM sodium phosphate buffer, pH 7.0) from a stock solution (1 mg nitrocefin dissolved in 500 µl DMSO and stored at –20°C). For one reaction use 20 µl diluted nitrocefin together with 5 µl translation plus 175 µl β-lactamase buffer in a 200 µl reaction.
7. Measure $OD_{486\,nm}$ immediately. Follow the kinetics for approximately 12 min, measuring at least once every minute.

2.7. Reagents for DARPin Expression and Binding Analysis of Single Clones (25)

1. *E. coli* strain XL-1 blue (Stratagene, No. 200268).
2. Expression plasmid pDST67 (20, 25), a derivative of pQE30 (QIAGEN).
3. 2×TY media: 5 g NaCl, 16 g tryptone, 10 g yeast extract per liter. Adjust pH to 7.2 with NaOH.
4. 96-well deep well plates (ABgene, No. AB-0661).
5. Tris-buffered saline (TBS): 50 mM Tris, 150 mM NaCl; adjust pH to 7.4 with HCl.
6. TBST: TBS containing 0.05% Tween-20.

7. 10% BSA in H_2O.
8. Mouse-anti-RGS$(His)_4$ antibody (QIAGEN, No. 34650).
9. Goat-anti-mouse IgG coupled to alkaline phosphatase (Sigma-Aldrich, No. A3562).
10. pNPP substrate (*p*-nitrophenyl phosphate disodium salt; Fluka, No. 71768): stock 1 M in pNPP buffer (50 mM $NaHCO_3$, 50 mM $MgCl_2$); aliquot and store at −20°C.
11. B-PER II detergent solution (Pierce, No. 78260).

3. Methods

3.1. Insertion of the Library

The ribosome display vector pRDV is used to ligate the library of interest using gene-specific primers and insertion via the restriction endonuclease sites *Bam*HI and *Hin*dIII as indicated in Fig. 1 (16, 26). The general elements that need to be present in a ribosome display vector are the T7 RNA polymerase promoter sequence to initiate efficient transcription and a RBS for docking of the ribosome to initiate translation. The PCR fragment (between the primers T7B and tolAk, Fig. 1) that serves as the template for transcription ends without a stop codon in the ORF. At both the 5′ and 3′ ends of the mRNA, stabilizing stem loops are incorporated to protect the mRNA from exonuclease degradation (2, 27). The absence of a stop codon in the resulting mRNA prevents termination of translation. The fact that the library is fused in frame to a spacer (or tether) sequence (e.g., derived from the *E. coli tolA* gene) allows the nascent protein chain to exit the ribosome and fold outside of the ribosome. The original pRDV contains an N-terminal FLAG tag instead of an N-terminal Met-Arg-Gly-Ser-$(His)_6$ tag as shown here for the case of the DARPin libraries (16). Both tag variants lead to good initiation of *in vitro* translation and yield ternary complexes in good yields.

3.2. Transcription of PCR Products

1. To obtain a length-defined fragment of DNA as template for *in vitro* transcription, use the outer primers T7B and tolAk in the following PCR reaction to introduce the T7 RNA polymerase promoter sequence, RBS, the stabilizing 5′ and 3′ stem loops and the tolA spacer sequence:

   5.0 µl 10× Thermopol buffer

   2.0 µl dNTPs (final concentration 200 µM each)

   2.0 µl DMSO (final concentration 5%)

   0.5 µl T7B primer (final concentration 1 µM)

   0.5 µl tolAk primer (final concentration 1 µM)

5.0 µl library DNA [either of the initial library or of the amplified DNA after selection which has been ligated to pRDV (see Note 6)]

0.5 µl Vent DNA polymerase (2 U/µl)

Add to 50 µl H_2O.

2. Perform a hot start to increase specificity and use the following cycling parameters (see Note 7): 3 min at 95°C, 25 cycles: 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, final extension 5 min at 95 °C.
3. Verify the product on an agarose gel.
4. For *in vitro* transcription set up the following reaction on ice:

   20 µl 5× T7 polymerase buffer

   14 µl NTPs (final concentration 7 mM each)

   4 µl T7 RNA polymerase (20 U/µl)

   2 µl RNasin (40 U/µl)

   22.5 µl PCR product without further purification

   Add to 100 µl with H_2O.
5. Incubate the transcription for 2–3 h at 37°C (see Note 8).

3.3. Cleanup of Template mRNA for In Vitro Translation

1. In order to remove all impurities from the reaction, the RNA needs to be purified. This can be performed in two ways:

   Conventional protocol

   (a) A LiCl precipitation can be performed to purify the RNA product. For this purpose, add 100 µl ice-cold H_2O and 200 µl ice-cold 6 M LiCl to the 100 µl translation reaction and vortex.

   (b) Incubate on ice for 30 min, then centrifuge at 20,000 × *g* at 4°C for 30 min.

   (c) Discard the supernatant and wash the pellet with 500 µl ice-cold 70% EtOH ensuring that the pellet is not disturbed.

   (d) Remove supernatant and dry pellet in a Speedvac apparatus.

   (e) Completely dissolve the pellet in 200 µl ice-cold H_2O and centrifuge at 20,000 × *g* at 4°C for 5 min to remove remaining precipitates.

   (f) Transfer 180 µl supernatant to a new tube without disturbing the pellet. Add 20 µl 3 M NaOAc and 500 µl ice-cold 100% EtOH, vortex.

   (g) Incubate at –20°C for at least 30 min. Vortex and centrifuge at 20,000 × *g* at 4°C for 30 min and discard the supernatant.

   (h) Wash the pellet with 500 µl ice-cold 70% EtOH, dry the pellet in a Speedvac apparatus, and resuspend the pellet in 30 µl H_2O.

Alternative protocol

(a) For purification of the RNA, small gel filtration columns (e.g., illustra MicroSpin™ G-50 Columns) can be used.

(b) Vortex the column to resuspend the material and break off bottom of the column.

(c) Place the column into a 1.5-ml tube and spin down at 735 × *g* for 1 min to pack the column material.

(d) Place the column into a collection tube, apply 50 µl sample from the transcription reaction, and centrifuge at 735 × *g* for 1 min.

(e) Optional: DNAse I treatment before loading the column (see Note 9): Take 43 µl of the transcription reaction and add 2 µl of DNAse I solution (10 U/µl) plus 5 µl 10× dilution buffer supplied with the enzyme. Incubate for 10–15 min at room temperature, and then apply the sample to the column.

2. Aliquot RNA and immediately freeze in liquid nitrogen. Store at –80°C.

3. Determine the RNA concentration of a 1:100 dilution by $OD_{260\,nm}$. If the transcription worked well, a yield of 3–8 µg/µl for RNA after LiCl/EtOH precipitation (total yield from a 100 µl reaction: 90–240 µg) or 1–3 µg/µl from the illustra MicroSpin™ G-50 Columns (total yield from a 50 µl reaction: 50–150 µg) should be obtained.

3.4. In Vitro Translation

1. For one *in vitro* translation reaction, set up the following mix on ice:

   2.0 µl 200 mM methionine

   41 µl premixZ with optimized composition

   x µl *in vitro* transcribed RNA (total 10 µg, volume follows from RNA concentration; see Note 10)

   50 µl S30 extract and add to 110 µl with H_2O (for preparation of the S30 extract, see Subheading 3.12)

   Add 0.625 µl PDI if your library scaffold requires the formation of disulfide bonds.

2. Mix carefully by pipetting up and down and incubate the reaction at 37°C for 10 min, the time found optimal for DARPins. The incubation time and temperature must be optimized for each library based on different constructs.

3. Stop the reaction by addition of 440 µl ice-cold STOP mix.

4. Mix by pipetting up and down and centrifuge at 20,000 × *g* at 4°C for 5 min. Transfer 500 µl supernatant to a fresh tube and use 100 µl per well when performing selection in plates or 250 µl per tube when performing selections in solution for either the target-containing or control reaction (see Subheading 3.5).

3.5. Selection (See Note 11)

3.5.1. Target Protein Preparation

Express and purify the target by methods of your choice. To immobilize the target for capturing the ternary complexes it is recommended to biotinylate the target. This is the method of immobilization found to be most robust by far to stringent washing, including washing with detergents. The advantage of immobilizing biotinylated targets is that it is very general, and it works equally well for proteins, peptides, oligonucleotides, and small molecules. Furthermore, by avoiding any direct binding to plastic surfaces, the structure of the target is maintained. Finally, the nonbiotinylated version of the target is a convenient competitor in off-rate selections and in the specificity screening of single clones. Biotinylation can be achieved in two ways (see Note 12):

1. Fuse the target to an AviTag and biotinylate it *in vivo* or *in vitro* using the *E. coli* biotinylation enzyme BirA (21) following the guidelines posted on the Avidity webpage (http://www.avidity.com).
2. Alternatively, biotinylate surface lysine amino acid residues using NHS-biotin reagents from Pierce following the manufacturer's instructions.

3.5.2. Selection in Plates

1. Coat wells of a 96-well Maxisorp plate with 100 µl of a 66 nM neutravidin or streptavidin solution in TBS and close with an adhesive plate sealer (see Notes 13 and 14). Store overnight at 4°C or for 1 h at room temperature. Invert the plate and shake out the solution, dry on paper towels, and wash the wells three times with 300 µl TBS.
2. Block the wells with 300 µl 0.5% BSA in TBST per well, seal and incubate on an orbital shaker for 1 h at room temperature. Shake out blocking solution and dry on paper towels.
3. Immobilize 100 µl biotinylated target at a concentration of 100–200 nM (can be decreased in later rounds) in TBST/0.5% BSA and TBST/0.5% BSA only for control wells. Seal and incubate on an orbital shaker at 4°C for 1 h. Wash the plate three times with 300 µl ice-cold TBST and once with 300 µl ice-cold WBT. Remove WBT only when the stopped translation reaction can be added to the wells (see Subheading 3.4).
4. Add the stopped *in vitro* translation, seal the plate, and incubate the binding reaction at 4°C for 1 h. Wash the wells with 300 µl ice-cold WBT containing 0.1% BSA for eight to ten times. Use two fast washes removing the buffer immediately, followed by incubations starting at 5 min and extending to 15 min in later rounds. In these longer incubations binders with fast off-rates will dissociate and subsequently be washed away.

5. For elution of the RNA, add 100 µl EB containing EDTA to release the mRNA from the captured protein–mRNA–ribosome complexes and freshly added *S. cerevisiae* RNA (final concentration 50 µg/ml) to block the surface of the tubes and perhaps to act as competing substrate for any residual RNases. Incubate at 4°C for 10 min and add to 400 µl lysis buffer of the High Pure RNA purification kit on ice. Repeat the elution step and collect the second elution in the same tube. After vortexing the RNA is stable and can be processed at room temperature until elution from the column (see Subheading 3.6).

3.5.3. Selection in Solution

1. Starting from the stopped and centrifuged *in vitro* translation reaction (see Subheading 3.4), divide the reaction into two aliquots of 250 µl and add 250 µl of STOP mix. Add 40 µl of streptavidin-coated magnetic beads that were washed two times with 500 µl TBS and blocked with 500 µl TBST/0.5% BSA for 1 h in a 2-ml tube as preselection step (see Note 14). Rotate at 4°C for 1 h.
2. Transfer the supernatant to a blocked 2-ml tube and add to 100–200 nM of biotinylated target (omit target in the control reaction) and incubate rotating at 4°C for 1 h (see Note 15).
3. Transfer the supernatant to a blocked tube containing 40 µl of streptavidin-coated magnetic beads and capture the ternary complexes rotating at 4°C for 30 min. Wash with 500 µl ice-cold WBT containing 0.1% BSA as indicated above (see Subheading 3.5.2). Separate captured complexes using a magnetic separator between each washing step.
4. Proceed with the elution and purification of RNA as described for the selection on plates (see Subheading 3.5.2).

3.5.4. Affinity Maturation by Competition with Non-labeled Target (Off-Rate Selection)

To increase the affinities of the library members, it is best to select for those having the lowest dissociation rate constant (off-rate) from the target (7, 13, 15, 28, 29). This off-rate selection can be applied for the improvement of known binders (after mutagenizing the gene for defined binders and thus creating a new library), but also during the initial selection from the original library. In this off-rate selection step an excess of nonbiotinylated target is added after the binding reaction has already been equilibrated for 1 h. Any fast dissociating binder will be immediately occupied by nonbiotinylated target and thereby prevented from being captured with biotinylated target on streptavidin or neutravidin. Conversely, any high-affinity binder with a slow off-rate will retain its biotinylated target and thus can be captured. The optimal duration of competitor incubation and the excess concentrations depend on the expected off-rates. As a general guideline, for the first off-rate selection a 2-h incubation with a 10 to 100-fold excess of competitor is recommended, and a 14-h incubation

with a 1,000 to 10,000-fold excess of competitor in later rounds may be appropriate. Then proceed with washing and elution of the bound ternary complexes as above.

3.6. Recovery of Eluted RNA

1. Apply the lysis buffer/eluate mixture on the column of the High Pure RNA isolation kit (see Note 16; *Optional:* as a positive control also purify 2 µl of the input RNA from the *in vitro* transcription diluted in 200 µl EB) and centrifuge at 8,000 × *g* for 1 min. Discard the flow-through.
2. Add 100 µl diluted DNAse I solution (1.8 U/µl) directly onto the column filter and incubate at room temperature for 15 min (see Note 17). Add 500 µl wash buffer 1 and centrifuge at 8000 × *g* for 1 min. Discard flow-through.
3. Wash with 500 µl wash buffer 2, centrifuge and discard flow-through.
4. Add 100 µl wash buffer 2 and centrifuge at 13,000 × *g* for 2 min to remove any residual EtOH.
5. Elute with 50 µl elution buffer and incubate for 2 min before centrifugation at 8,000 × *g* for 1 min into a fresh 1.5-ml RNAse-free tube.
6. Freeze the remaining sample of eluted RNA in liquid nitrogen and store at –80°C (see Notes 10 and 18).

3.7. Reverse Transcription of DARPin mRNA

1. Transfer two times 12.5 µl of eluted RNA to fresh 1.5-ml tubes (see Note 19).
2. Denature the eluted RNA at 70°C for 10 min and chill on ice.
3. Set up the following reverse transcription (RT)-mix (total of 7.75 µl) per RT reaction on ice:
   - 0.25 µl WTC4 primer (final concentration 1.25 µM)
   - 0.5 µl dNTPs (final concentration 125 µM of each nucleotide)
   - 0.5 µl RNasin (40 U/µl)
   - 0.5 µl AffinityScript™ Multiple Temperature Reverse Transcriptase (50 U/µl)
   - 2.0 µl 10× AffinityScript buffer
   - 2.0 µl DTT (final concentration 10 mM)
   - 2.0 µl H_2O
4. Distribute 7.75 µl RT-mix per RT reaction to the 12.25 µl samples of denatured RNA.
5. Incubate at 50°C for 1 h.
6. Use 2–5 µl as template for PCR using the inner primers WTC4 and EWT5s.
7. Freeze the rest of the cDNA in liquid nitrogen and store at –20°C.

3.8. Amplification of cDNA Coding for DARPins

The standard protocol for Vent DNA polymerase (NEB) is shown below. If another DNA polymerase or primers are used, the reaction conditions might have to be adapted.

1. Set up the following reaction mix per sample:
   - 2–5 µl cDNA
   - 5.0 µl 10× Thermopol buffer (NEB)
   - 2.0 µl dNTPs (final concentration 200 µM of each nucleotide)
   - 2.5 µl DMSO (final concentration 5%)
   - 0.5 µl WTC4 primer (final concentration 1 µM)
   - 0.5 µl EWT5s primer (final concentration 1 µM)
   - 0.5 µl Vent DNA Polymerase (2 U/µl)
   - Add to 50 µl with H_2O
2. Perform a hot start PCR to reduce unspecific amplification. Use the following cycling parameters: 3 min at 95°C, 25 cycles: 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, and final extension 5 min at 95°C (see Note 20).
3. Verify the product on an agarose gel (see Note 21).

3.9. Incorporation of Promoter Elements, RBS, tolA Spacer and RNA-Stabilizing Stem Loops

1. Purify PCR products either by excision of the according bands from the agarose gel and subsequent purification or direct purification over commercially available columns, for example, of the NucleoSpin extract II kit. Elute in a small volume of 20 µl.
2. Digest≥150 ng of the PCR product with the corresponding restriction enzymes, e. g., *Nco*I and *Hin*dIII for DARPin selections, in a final volume of 30 µl at 37°C for 2 h (see Note 22).
3. Purify digested PCR product using the NucleoSpin® Extract II DNA purification kit. Elute in 15 µl elution buffer supplied with the kit.
4. Ligate the PCR fragments into the ligation-ready pRDV plasmid using 100 ng of digested pRDV and the digested PCR product with a molar ratio of vector to insert of 1:5–7 in a final volume of 10 µl. Add 1 U of T4 DNA ligase and 1 µl ligase buffer. Incubate 30–60 min at room temperature. Use this ligation as PCR template with the T7B and tolAk primers (see Subheading 3.2) or perform an error-prone PCR to increase diversity (see Subheading 3.10).

3.10. Error-Prone PCR

1. Set up reactions introducing different mutational rates using various concentrations in the range of 1–20 µM of the nucleotide analogs dPTP and 8-oxo-dGTP (see Note 23): 10 ng pRDV_DARPin template, 250 µM dNTPs each, 1 µM T7B primer and tolAk primer, 1× polymerase buffer, 1.5 mM $MgCl_2$ and 0.2 µl Platinum® Taq DNA Polymerase in a 50 µl reaction.

2. Apply the following cycling parameters (must be adapted according to primers and template): 3 min at 95°C, 25 cycles: 30 s at 95°C, 30 s at 50°C, 1 min at 72°C, and final extension 5 min at 95°C.
3. Verify the product on an agarose gel.
4. Mix PCR products in equimolar amounts to serve as template for the *in vitro* transcription (see Subheading 3.2, step 4).

3.11. Initial Analysis of Selected Individual Library Members in a 96-Well Format (22)

1. After RT-PCR (see Subheadings 3.7 and 3.8) prepare the DARPin pool after enrichment has been observed for subcloning into a prokaryotic expression plasmid using the endonucleases *Bam*HI and *Hin*dIII. Enrichment is indicated by a much stronger PCR band recovered from a well with immobilized target than from a control well without immobilized target.
2. Ligate the PCR fragment into pDST67 (20, 25) as fusion with the sequence coding for a N-terminal $MRGS(H)_6$ tag for purification.
3. After transformation into *E. coli* XL1-Blue pick single clones and inoculate in deep 96-well plates in 1 ml 2×TY/1% glucose/amp (100 µg/ml) grow overnight at 37°C while shaking at 540 rpm on an orbital shaker.
4. Transfer 100 µl of each culture to 900 µl fresh media and grow at 540 rpm for 1 h at 37°C.
5. Induce with 0.5 mM IPTG (add 100 µl media containing 5.5 mM IPTG) and grow an additional 3–5 h at 37°C.
6. Harvest cells by centrifugation at 400×*g* for 10 min, and discard supernatant.
7. Resuspend pellet in 50 µl B-PER II detergent and lyse cells for 15–30 min on an orbital shaker at 500 rpm.
8. Add 1 ml TBST/0.1% BSA and centrifuge to remove debris.
9. Use 10 µl (using a predilution of 1:100 in TBST/0.1% BSA can give you a better indication of the affinity of the binders when they are in the µM to low nM range) for ELISA. Binders with even higher affinity will still fully saturate the immobilized target and can only be distinguished in ELISA by inhibition with low concentrations of soluble target.
10. For ELISA, coat wells with 100 µl of 66 nM neutravidin in TBS for 1 h at room temperature or overnight at 4°C. Wash two times with 300 µl TBS. Dry plate on paper towels after each step.
11. Block with 300 µl TBST/0.5% BSA for 1 h at room temperature.
12. Invert plate and shake out liquid and immediately add 100 µl of the biotinylated target (10–100 nM) in TBST/0.1% BSA. Incubate 1 h at 4°C or room temperature on an orbital shaker (see Note 24). Wash three times with 300 µl TBST.

13. Add 100 µl DARPin extract from step 9. Incubate 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µl TBST.
14. Add 100 µl mouse-anti-RGS(His)$_4$ antibody in a 1:500 dilution. Incubate 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µl TBST.
15. Add 100 µl goat-anti-mouse antibody coupled to alkaline phosphatase in a 1:20,000 dilution. Incubate 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µl TBST.
16. Add 100 µlpNPP substrate solution. Incubate until color development at 4° to 37°C depending on the stability of the target and the library scaffold and determine $OD_{405\ nm}$.

3.12. Preparation of S30 Extract (30–32)

1. Grow a 100-ml culture of *E. coli* MRE600 in incomplete rich medium overnight at 37°C.
2. Transfer 10 ml of the overnight culture in 1 l of fresh media in a 5-l baffled shaker flask and grow until $OD_{600\ nm}$ of 1.0–1.2 at 37°C while shaking at 230 rpm. This procedure can be scaled up to your needs, and 1 l culture usually yields 10–15 ml of S30 extract. The S30 extract is stable for years when stored at –80°C.
3. Chill cultures for 10 min on an ice water bath with gentle shaking.
4. Centrifuge cells at 3,500 × *g* at 4°C for 15 min and discard supernatant.
5. Wash the pellet three times with 50 ml of ice-cold S30 buffer per 1 l culture. It is best to resuspend cells with plating beads or on a magnetic stirrer using a sterile stir bar.
6. Freeze the cell pellet in liquid nitrogen and store for a maximum of 2 days at –80°C or continue immediately.
7. Resuspend the cell pellet (use 50 ml ice-cold S30 buffer per 1 l of culture), centrifuge at 4,000 × *g*. Discard supernatant and resuspend pellet in 4 ml S30 buffer per g wet cells (typically 1 l of culture yields 1.5–2.0 g cell pellet).
8. Lyse the cells by one single passage through a French press applying 1,000 psi or an EmulsiFlex at ~17,000 psi.
9. Centrifuge cells at 20,000 × *g* (SS-34) at 4°C for 30 min. Transfer supernatant to clean centrifuge bottle(s) and repeat this step.
10. Add 1 ml of preincubation mix to each 6.5 ml of cleared supernatant (usually 1 l culture will yield 8–10 ml of S30 extract) and slowly shake at 25°C for 1 h. In this time all

endogenous mRNA will be translated and cellular nucleases will degrade mRNA and DNA (33).

11. Dialyze the S30 extract in a tubing with a MW cutoff of 6,000-8,000 Da (Spectrum Laboratories SpectraPor No. 132650) against a 50-fold volume of S30 buffer at 4°C three times for 4 h.
12. Centrifuge S30 extract at 4,000 × *g* at 4°C for 10 min. If the library members and target are devoid of disulfide bonds, 1 mM DTT can be added to the extract. Aliquot at 4°C in suitable volumes (e.g., 55 µl is sufficient for one *in vitro* translation reaction, 110 µl for two) since it should not be refrozen to guarantee best activity. Flash freeze in liquid nitrogen and store at –80°C.

4. Notes

1. Use RNAse-free water, chemicals, and consumables. Most commercially available water is RNase-free or can be generated using a membrane microfiltration system, e.g., MilliQ from QIAGEN, to produce ultrapure water. Alternatively, you can use 0.1% DEPC (diethylpyrocarbonate), which reacts with histidine residues but also other nucleophilic groups and therefore inactivates RNases, but for the same reason it cannot be used, for example, with Tris-buffers. Chemicals should be kept separate from the common chemical shelf and handled only with gloves and a flamed spatula to avoid RNase contamination. Purchase only RNase-free plastic consumables. If necessary you can bake glass bottles and pipettes at 180°C for 6 h.
2. The buffer composition may be adjusted to the requirements of the library and target, but it is important that the wash buffer contains 50 mM Mg^{2+} to stabilize the ribosome. It is recommended to test buffer conditions with a known binder to ensure stability of the nascent chain complex.
3. Different reverse transcriptases [AffinityScript™ Multiple Temperature Reverse Transcriptase, SuperScript™ II (Invitrogen, No. 18064-022), ThermoScript™ (Invitrogen, No. 12236-014), and QuantiTect (QIAGEN, No. 205310)] were tested for efficiency on DARPin sequences. With exception of QuantiTect the yield obtained was comparably high with all other reverse transcriptases.
4. Previously Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, No. F-530S) has also been used (26, 30). Different DNA polymerases were tested [$Vent_R$® DNA Polymerase, Herculase® II Fusion DNA Polymerase (Stratagene,

No. 600677), Expand High Fidelity PCR System (Roche Diagnostics, No. 11732641001]. The DNA polymerase mix from the Expand High Fidelity PCR System gave the lowest yield of PCR product, while Herculase II gave the highest amount of side products. Therefore, we now routinely use $Vent_R^{®}$ DNA Polymerase for amplification of DARPin sequences. Since the yield was highest with the Herculase II it might be a good alternative to increase the yield of PCR product or for amplification of other library scaffolds.

5. Use the home-made RNA polymerase buffer (see Subheading 2.4) as indicated for maximum yield of RNA. Commercial buffers have not worked very well at this step when the PCR product is directly used without further purification.
6. In round one ensure that the number of molecules actually exceeds the library size. Conversely, in a newly constructed library, the diversity cannot be higher than the number of molecules used in this step. In later rounds, an enrichment is obtained, and it is generally sufficient to use ~50 ng of pRDV_DARPin template.
7. The PCR products can be used without additional purification.
8. Optionally, the transcribed RNA can be analyzed on a denaturing formaldehyde agarose gel following standard procedures (34). The mRNA product should give a sharp band. A smear or no product indicates RNase contamination, which needs to be eliminated and the step repeated. If the band is sharp but the yield is lower than expected: Obtain more starting DNA template by not purifying the PCR product that is used as template, as the quality is usually sufficient even without purification, and do use the home-made RNA polymerase buffer (Subheading 2.4) for better transcription yield. If the products are not of the expected size, optimize the PCR conditions depending on your template and primers.
9. In some cases the template DNA might bind unspecifically to the target, for example, if the target is highly positively charged, and then it is recommended to remove this contamination by DNAse I treatment before the actual selection. Always freeze small aliquots of DNAse I and store at –20°C. Do not refreeze or vortex solutions containing DNAse I, because the enzyme is very sensitive to denaturation.
10. Always freeze RNA immediately after use and only thaw when needed to avoid degradation.
11. For the selection, some general considerations need to be pointed out. Always use the same target preparation through all of the selection and screening rounds, and ensure its quality and account for its stability over the duration of the experiment. If the target denatures, epitopes present in the native

protein will vanish, and such binders will be lost. Account for high diversity especially in the first round by using sufficient starting library. Start selections with a higher number of DNA template molecules than the diversity of the library. To extract all putative binders in the library use a larger surface area to immobilize the target in the first round. The first round should, in general, not be highly selective; it is more important to capture the full diversity of binders, as a binder lost at this stage can never be recovered. In general, it is recommended to perform the selection in duplicates to monitor the selection quality. It is recommended to switch between neutravidin (a chemically modified derivative of avidin) and streptavidin, or even switch between selections on immobilized target and target in solution during the selection process, to focus selection on binding to the target, rather than on streptavidin/neutravidin or any other surface features. If high-affinity binders in the pM range are needed, include the introduction of additional random mutations using error-prone PCR and increase stringency by applying off-rate selections. Perform one cycle of nonstringent selection including an error-prone PCR followed by a round of off-rate selection without error-prone PCR (see Subheading 3.5.4). The rationale is that error-prone PCR will generate many nonfunctional molecules. First, *all* functional molecules should be recovered by a nonstringent selection, then from this pool of functional (randomized) molecules, the best ones should be recovered. Use these to perform a stringent round using off-rate selection. Start at 10–100-fold excess competitor; increase to 100–10,000-fold in later rounds, if feasible (35). These two rounds should be followed again by a nonstringent round without any additional selection pressure; simply to amplify the rare molecules. Perform this cycle of error-prone PCR, off-rate selection, and nonstringent round two to three times before analyzing single clones (see Subheading 3.10).

12. Using the AviTag has the advantage that all biotinylated proteins are labeled uniformly and remote from epitopes, which might interfere with later use and are labeled only once, leading to a more homogenous target preparation. Avoid the presence of a Met-Arg-Gly-Ser-$(His)_6$ tag ("RGS-His-tag") on the biotinylated target, rather use a $(His)_6$ tag for purification, since the detection of DARPins bound to the target is performed using an anti-RGS$(His)_4$ antibody (see Subheading 3.10). Make sure your target sample is devoid of free biotin. Biotin removal requires an extensive dialysis, for example, four times against an 100-fold volume buffer for 4 h each. Nonbiotinylated target can be removed using a monomeric avidin column following the manufacturer's instructions (Pierce, No. 53146).

13. Use one well as nontarget control and two wells with immobilized target in later rounds as mutual controls for enrichment. When starting from the libraries in round 1 it is recommended to use a larger surface, for example, four wells with immobilized target.
14. To remove unspecifically binding ribosomal complexes it is recommended to use a preselection on BSA-blocked wells coated only with neutravidin or streptavidin, but omitting the target protein, except for round 1, where this "prepanning" should not be done. For prepanning, the preparation of additional wells and incubation of the ternary complexes from the *in vitro* transcription for 30–60 min are necessary before transferring the solution to the target-coated or control wells.
15. The amount of target can be reduced to 100 pM, for example, when performing an off-rate selection, and thus a high amount of competitor can be added. At even lower target concentrations the unspecific binding might dominate over target binding, however, and thus specificity of binding must be carefully controlled.
16. RNA isolation can also be performed with the RNeasy mini kit (QIAGEN, No. 74104) with comparable yield of resulting PCR product.
17. This step is highly recommended to avoid amplification of nonselected template DNA that has been carried over through all steps of the selection procedure. See also Note 9 for handling of DNAse I.
18. The RNA should be stable for years at –80°C, but we recommend to immediately proceed with cDNA synthesis and PCR amplification for best recovery of sequences of putative binders.
19. Use one sample without addition of reverse transcriptase as control. The result of the following PCR will be a measure for the quality of the selection regarding DNA carry-over from the input DNA and putative overcycling (see Notes 17 and 20).
20. Depending on the round of selection more or fewer cycles could be advantageous. In the first round, 32–40 cycles are recommended to obtain sufficient product. After more rounds of selection specific binders are being enriched, therefore, the output of eluted RNA molecules increases. By lowering the cycle numbers in round 2 to between 28 and 35 and in all following rounds to 25, unspecific amplification can be reduced to a minimum. In addition, note that when the selection pressure increases, for example, after off-rate selection, the yield of PCR product might decrease. In this case use more cycles.
21. If the quality and amount (<10 ng/µl) of the PCR product was not satisfactory repeat the PCR. Never reamplify the PCR product, because this might lead to unspecific amplification of unwanted by-products.

22. In parallel, digest the ribosome display vector (pRDV) with the same restriction enzymes, for example, *NcoI* and *Hin*dIII for DARPin selections. Purify the plasmid backbone using extraction from a preparative agarose gel. It is recommended to use a larger preparation to last for several selection rounds and/or multiple target selections: Test the quality of the digested plasmid by ligation and transformation and/or PCR on the ligation mix to evaluate the level of religation and therefore quality of the ligation-ready plasmid.
23. Add different concentrations of the nucleotide analogs, for example, 0, 3, and 10 μM. Up to 20 μM can be used, but the amount of product is greatly reduced at this concentration. The mutational load per kb under the conditions described is 1.5 mutations with 1 μM nucleotide analogs and 3.2 mutations with 3 μM nucleotide analogs using Platinum Taq polymerase. These numbers refer to fresh nucleotides and can vary if the nucleotides are no longer incorporated well, for example, by hydrolysis of the triphosphate. The use of a low to medium mutational load per selection, but repeating over several rounds, might be beneficial over a high mutational load which might result in a high number of misfolded library members in the pool.
24. The incubation temperature depends on the stability of the target and library scaffold.
25. Some applications, e.g., for therapy, require high stability of the therapeutic agent (36). Selection for high stability can also be achieved with ribosome display. This is best achieved by first making the whole population unable to fold, by introducing a reversible destabilization, and then selecting for compensating mutations, and finally removing the destabilization again. For example, most antibody domains require disulfides for stability, which form only under oxidizing conditions. A destabilization and increase in aggregation of the antibody fold is usually observed when the disulfides are removed (6, 37). Using a reducing environment during the selection, scFv antibody fragments could be evolved that were able to fold under reducing conditions correlating with conditions in the cytosol, and they showed higher stability than the starting molecule in the absence of the disulfide bonds (7), but also after the disulfide bonds were allowed to form again. Antibody fragments with these improved biophysical property can be used in biomedical applications with disulfides formed, but they also make an intracellular application (as “intrabodies”) (38) more feasible. In addition, rational design of the antibody framework (39) could contribute to the development of stability-improved, antibody-based therapeutics.

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